(For Laboratory Use Only)

ATS Labs Project #_____

ATS LABS

PROTOCOL

Carpet Sanitizer

Test Organism:

Staphylococcus aureus (ATCC 6538)
Enterobacter aerogenes (ATCC 13048)

PROTOCOL NUMBER

RUG01072712.CSAN

PREPARED FOR

Rug Doctor, Inc.
415C Axminister Drive
Fenton, MO 63026-2497

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PREPARED BY

Becky Lien, B.A.
Research Scientist I

DATE

July 27, 2012

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Carpet Sanitizer

SPONSOR: Rug Doctor, Inc.
415C Axminister Drive
Fenton, MO 63026-2497

TEST FACILITY: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PURPOSE

The purpose of this study is to determine the effectiveness of the Sponsor's product as a carpet sanitizer following the EPA Carpet Sanitizer methodology. This method is in compliance with the requirements of The U. S. Environmental Protection Agency (EPA).

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is August 10, 2012. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of September 7, 2012. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, due to failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs or any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the United States EPA concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

The U.S. Environmental Protection Agency requires that products bearing label claims for effectiveness as sanitizers for pre-cleaned carpeting must be supported by appropriate scientific data demonstrating the efficacy of the test substance against the claimed organism. This is accomplished by treating the target test organism with the test substance under conditions which simulate as closely as possible, in the laboratory, the actual conditions under which the test substance is designed to be used. The experimental design in this protocol meets these requirements.

TEST PRINCIPLE

Test organism cells dried on carpet squares are exposed to the test substance for a specified exposure time. After exposure, the carpet squares are transferred to vessels containing neutralizer and assayed for survivors. Appropriate population controls, culture purity, sterility and neutralization confirmation controls will be performed. The current revision of standard operating procedure CGT-4137 reflects the methods which shall be used in this study.

TEST METHOD

Chart 1:

Test Organisms	ATCC #	Growth Media	Incubation Parameters
Staphylococcus aureus	6538	Nutrient Agar A & B	35-37°C, aerobic
Enterobacter aerogenes	13048	Nutrient Agar A & B	25-30°C, aerobic

The test organisms to be used in this study were obtained from the American Type Culture Collection (ATCC), Manassas, VA.

Neutralizer: Neutralizing broth appropriate for the test substance.

Carriers

Two carpet types, nylon and/or polypropylene carpet are recommended for use. Alternate carpet types may be tested, where appropriate. The Sponsor will be responsible for selecting the appropriate carpet to be used in the test. The carpet selected for testing will be cut into approximately 8 inch x 12 inch pieces. Six approximately 2 inch x 2 inch square carriers will be cut into the carpet. The carpet will be fastened to a mounting tray (or equivalent) and will be autoclave sterilized for ≥ 20 minutes prior to use in testing.

Preparation of Test Organism

The test organism will be transferred daily on Nutrient Agar A slants for ≥ 3 but ≤ 30 transfers. Wash the growth from a 24 ± 2 hour Nutrient Agar A slant using 5.0 mL of phosphate buffer dilution water (PBDW). The 0.01% Triton has been omitted from the diluent to avoid the potential for interaction with the test substance. Aspirate the growth and transfer the growth to 99 mL PBDW. A 2.0 mL aliquot of this suspension will be added to sufficient Nutrient Agar B bottles. The inoculum will be evenly distributed within the bottles and the excess inoculum will be removed. Incubate the bottles, agar side down, for 18-24 hours at the incubation conditions listed in chart 1. Following incubation, approximately 3 mL of PBDW will be added to each bottle and the inoculum will be collected. Approximately 15-20 sterile glass beads will be added to the bottles to aid in recovery. -The growth suspension will be removed and filtered through sterile gauze or sterile Whatman #2 filter paper pre-wetted with 1.0 mL of PBDW. Similar to AOAC 960.04 to ensure that any agar harvested with the organism is removed from the test suspension, the growth suspension will be filtered through sterile gauze or Whatman #2 filter paper pre-wetted with 1.0mL of PBDW. The test culture may be further adjusted, where appropriate, to target approximately $1x10^{8}$- $1x10^{10}$ CFU/mL. The bacterial target range is recommended based on ATS' history of conducting the method to assure that the Unscrubbed population control achieves 1 x 10^{6} CFU/carrier. McFarland standards or a spectrophotometer may be used to aid with culture adjustment. An organic soil load may be added per Sponsor request.

Contamination of Carriers

Inoculate each cut square with 100 µL of the prepared test culture using a calibrated pipettor. To aid in drying and carrier uniformity, the inoculum will be spread over the upper surface of the carpet using a sterile loop. The inoculated carpet will be dried in an incubator at 35-37°C for 60 minutes with sterile foil loosely covering the carpet.

Preparation of Test Substance

The test substance(s) to be assayed will be used as directed by the Sponsor. If a dilution of the test substance is requested by the Sponsor, the diluted test substance(s) shall be used within three hours of preparation.

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Exposure Conditions
After drying, apply the test substance as specified by the Sponsor. Scrub each carpet carrier for approximately 30 seconds using approximately 30 circular clockwise strokes and approximately 30 circular counterclockwise strokes using a 4¼ x 1 5/8-in. surgical hand brush with ½-in bristles. A circular area of pile approximately 3 inches (7.6 cm) in diameter around the center of each carrier will be scrubbed using this treatment. Moderate to heavy pressure will be applied downward on the brush to work the solution to the base of the pile. A new sterile brush will be used for each carpet square. A calibrated timer will be started upon application of the test substance and staggered intervals will be followed to treat subsequent carpet carriers. The treated and scrubbed carpet will remain at room temperature, uncovered, for the Sponsor specified exposure time. Exposure begins once the test substance has been applied.

Test System Recovery
Following the Sponsor specified exposure time, remove each carrier from the larger carpet piece. Transfer each carpet carrier to individual vessels containing approximately 100 mL neutralizer broth and 10 stainless steel penicylinders; this represents a 10^0 dilution. Care will be taken to place the carrier carpet side down within the neutralizer vessel. Each vessel will be shaken for at least 1 minute, at approximately 200 RPM, to free the bacteria from the carpet fibers. Ten-fold serial dilutions will be prepared. A 1.0 mL aliquot of the $10^0 - 10^{-3}$ dilutions will be plated in duplicate onto the appropriate agar. *If swarming is a concern, 1.0 mL of 10^0 will be plated in duplicate. In addition, 0.1 mL of $10^0 - 10^{-2}$ will be plated in duplicate.*

Incubation and Observation
Incubate *S. aureus* plates at 35-37°C for 48 ± 4 hours. Incubate *E. aerogenes* plates for 48±4 hours at 25-30°C. If necessary, the subcultures may be placed at 2-8°C for up to three days prior to examination.

Following incubation, the subcultures will be visually examined for growth. If possible, count plates containing between 30 and 300 CFU.

Representative test plates will be stained and/or biochemically assayed to confirm or rule out the presence of the test organism.

STUDY CONTROLS

Suspension Population Control
Ten-fold serial dilutions will be prepared using a 1.0 mL sample of this starting test culture. A 1.0 mL aliquot of the 10^5-10^9 dilutions will be plated in duplicate onto the appropriate agar.

Population Controls
Inoculate and dry six carpet carriers, per test organism, per carpet type as in the test procedure.

Unscrubbed Population Control
Allow three of the six inoculated population control carpet squares to stand, untreated for the exposure time. Following exposure, subculture and mix each individual carpet carrier as indicated in the test procedure. Prepare ten-fold serial dilutions and plate 1.0 mL of 10^{-1} through 10^{-4} in duplicate for each carrier. If swarming is a concern, plate 0.1 mL of 10^0 through 10^{-3} in duplicate for each carrier. Incubate the plates as in the test. The acceptance criterion for this study control is an average minimum of 1.0×10^6 CFU for the unscrubbed carpet squares.

Scrubbed Population Control
The remaining three inoculated population control squares will be treated with sterile deionized water containing 0.01% Triton X-100 and will be scrubbed as in the test procedure. Following exposure, subculture and mix each individual scrubbed carpet carrier as indicated in the test procedure. Prepare ten-fold serial dilutions and plate 1.0 mL of 10^{-1} through 10^{-4} in duplicate for each carrier. If swarming is a concern, plate 0.1 mL of 10^0 through 10^{-3} in duplicate for each carrier. Incubate the plates as in the test. The acceptance criterion for this study control is growth of the test organism.

Neutralization Confirmation Control
The neutralization of the test substance will be confirmed by exposing sterile carpet carriers to the test substance and scrubbing as in the test procedure. If multiple test substance concentrations and/or exposure time points are followed in the test, only the most concentrated test substance and/or shortest time point needs to be evaluated in this control. Following the Sponsor specified exposure time, transfer the carpet to the neutralizer as in the test. Challenge the neutralizer with a low level of the test organism to target approximately 100 CFU per mL of neutralizer. Mix and plate 1.0 mL of the neutralizer, in duplicate, incubate and observe for the presence of growth. A neutralization confirmation numbers control will be performed by inoculating untreated neutralizer with 1.0 mL of the diluted culture, and plating, as described above. NOTE: If swarming is a concern, 0.1 mL aliquots will be plated in duplicate. In this case, sufficient organism should be added to the vessels to target approximately 1000 CFU per mL of neutralizer. The plates will be incubated as in the test. The acceptance criterion for this study control is growth of the test organism in the neutralization confirmation within 1.0 $\log_{10}$ of the corresponding neutralization confirmation population control.

Purity Control
A "streak plate for isolation" will be performed on the organism culture and examined following incubation in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.

Organic Soil Sterility Control
A 1.0 mL aliquot of the soil used in testing will be added to a tube of Fluid Thioglycollate Medium and will be incubated as in the test. The acceptance criterion for this study control is lack of growth.

Carrier Sterility Control
A representative carpet carrier will be added to the neutralizer. A 1.0 mL aliquot of the neutralizer containing the carrier will be plated on Tryptic Soy Agar + 5% Sheep's Blood agar and the plate(s) will be incubated as in the test and examined for growth. This control is for informational purposes only.

Neutralizer Sterility Control
A 1.0 mL aliquot of representative, uninoculated neutralizer will be plated on Tryptic Soy Agar + 5% Sheep's Blood agar and the plate(s) will be incubated as in the test and visually examined. The acceptance criterion for this study control is lack of growth.

PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

ATS Labs maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including test organism strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subcultures, etc. during the course of the test. Test subcultures are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and/or macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: NA

STUDY ACCEPTANCE CRITERIA

Test Substance Performance Criteria
The U.S. EPA efficacy performance requirements for label claims state that the test substance must demonstrate a minimum 99.9% reduction of the test organism as compared to the scrubbed population control.

Control Acceptance Criteria
The study controls must perform according to the criteria detailed in the study controls description section. If any of the control acceptance criteria are not met, the test may be repeated under the current protocol.

REPORT

The report will include, but not be limited to, identification of the sample, date received, initiation and completion dates, identification of the organism strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185. A draft report will be provided to the Sponsor for review prior to finalization.

PROTOCOL CHANGES

If it becomes necessary to make changes in the approved protocol, the revision and reasons for changes will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

Standard operating procedures used in this study will be the correct effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.

TEST SUBSTANCE RETENTION

It is the responsibility of the Sponsor to retain a sample of the test substance. All unused test substance will be discarded following study completion unless otherwise indicated by Sponsor.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

REFERENCES

1. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, 1982. Subseries 91A, 91-4(b), Public Health Uses. In Pesticide Assessment Guidelines – Subdivision G (Product Performance).
2. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, 1982. Subseries 91A, 91-30 (d)(13), Public Health Uses. In Pesticide Assessment Guidelines – Subdivision G (Recommended Methods).
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-8, April 18, 1981.

DATA ANALYSIS

Calculations

Number of Organisms Surviving per Carrier

$$\text{CFU/carrier} = \frac{(\text{average CFU}) \times (\text{dilution factor}) \times (\text{volume neutralized solution in mL})}{(\text{volume plated in mL})}$$

The carrier population control will be calculated and reported using data from the most appropriate dilution(s).

Geometric Mean of Number of Organisms Surviving on Test or Control Carriers

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_N}{N}$$

Where: X equals CFU/carrier
N equals number of carriers

Percent Reduction

$$\% \text{ reduction} = [(a - b) / a] \times 100$$

where:
a = geometric mean of the number of organisms surviving on the population control carriers.
b = geometric mean of the number of organisms surviving on the test carriers.

Recovery Log_{10} Difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization Results)
Used for the neutralization confirmation control

Statistical Methods
None Used.

STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name and Batch Number - exactly as it should appear on final report):
(Lot codes for three lots (one of which will be >60 days old will be inserted.)

Expiration Date: **(Expiration Dates for all lots will be inserted)**

Product Description:

❑ Quaternary ammonia ❑ Peracetic acid ❑ Iodophor √ Peroxide
❑ Sodium hypochlorite ❑ Other_____

Test Substance Active Concentration (upon submission to ATS Labs):(Conc. will be inserted for all lots)

Neutralization/Subculture Broth:

❑ _____
✓ ATS Labs' Discretion. By checking, the Sponsor authorizes ATS Labs, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

Storage Conditions

✓ Room Temperature
❑ 2-8°C
❑ Other_____

Hazards

❑ None known: Use Standard Precautions
✓ Material Safety Data Sheet, Attached for each product
❑ As Follows:_____

Product Preparation

√ No dilution required, Use as received (RTU)
❑ *Dilution(s) to be tested:

_____ defined as _____ + _____
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

❑ Deionized Water (Filter or Autoclave Sterilized)
❑ Tap Water (Filter or Autoclave Sterilized)
❑ AOAC Synthetic Hard Water: _____PPM
❑ Other _____

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Carpet Type (Two carpet types will be selected.)

❑ Polypropylene/Olefin
❑ Nylon
√ Sponsor provided carpet - Polypropylene and Nylon
❑ Other _____

Test Organism(s)

☑ *Staphylococcus aureus* (ATCC 6538)
☑ *Enterobacter aerogenes* (ATCC 13048)

Carrier Number: 6 carpet squares per lot per carpet type per test organism

Application instructions (as applied to each 2"x 2" carrier); Hold the spray bottle approximately 3 inches from the surface of the carpeting and dispense around XX mLs of product per 2x2inch area to be sanitized.

Approximate Spraying Distance: 3 inches from the surface of the carpeting

Exposure Time: 60 Minutes **Exposure Temperature:** Room temperature

Organic Soil Load:

✓ Minimum 5% Organic Soil Load (Fetal Bovine Serum)
❑ No Organic Soil Load Required
❑ Other_____

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TEST SUBSTANCE SHIPMENT STATUS

- ☐ Has been used in one or more previous studies at ATS Labs.
- ☐ Has been shipped to ATS Labs (but has not been used in a previous study).
  Date shipped to ATS Labs:_____ Sent via *overnight* delivery? ☐ Yes ☐ No
- ☐ Will be shipped to ATS Labs.
  Date of expected receipt at ATS Labs:_____
- ☐ Sender (if other than Sponsor):_____

COMPLIANCE

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.

- ☑ Yes
- ☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

- ☐ Approved without modification
- ☐ Approved with modification

_____

_____

PROTOCOL ATTACHMENTS

Supplemental Information Form Attached - ☐ Yes ☐ No

APPROVAL SIGNATURES

SPONSOR:

NAME: Ms. Jennifer Bopp TITLE: Microbiologist, R&D

SIGNATURE:_____ DATE:_____

PHONE: (636) 717 - 2054 FAX: (636) 717 - 2047 EMAIL: Jennifer.Bopp@rugdoctor.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

_____

ATS Labs:

NAME: _____
Study Director

SIGNATURE:_____ DATE:_____
Study Director